Expression and Topography of Integrins and Basement Membrane Proteins in Epidermal Carcinomas: Basal but not Squamous Cell Carcinomas Display Loss of $\alpha6\beta4$ and BM-600/Nicein

Paola Savoia,* Livio Trusolino,* Eugenio Pepino,† Ottavio Cremona,* and Pier Carlo Marchisio*
*Dipartimento di Scienze Biomediche e Oncologia Umana, Università di Torino; and †Divisione di Dermochirurgia, Ospedale Dermatologico San Lazzaro, Torino, Italy

The expression and topography of some integrins and basement membrane proteins in cutaneous basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) have been studied by immunohistochemistry and Western blotting. It has been shown that the typical cell-to-cell distribution of $\alpha2\beta1$ and $\alpha3\beta1$ found in normal epidermis is replaced by pericellular distribution in both BCC and SCC cells. BCC and SCC also showed different patterns of expression of $\alpha6\beta4$, an integrin heterodimer normally lining the basal surface of basal epidermal keratinocytes: whereas SCC showed high expression and pericellular distribution of $\alpha6\beta4$, BCC cells did not express this integrin at all. The absence of $\alpha6$ and $\beta4$ subunits from BCC extracts was confirmed by Western blotting. The molecular composition of the basement membrane was markedly different in the two types of epidermal tumors. Whereas laminin and collagen type IV were conserved in the basement membrane zone of both tumors, the molecular complex BM-600/nicein, which is recognized by the monoclonal antibody GB3 and is possibly identical to the previously described basement membrane glycoproteins kalinin and epiligrin, was absent from BCC cells. Then, the simultaneous loss of expression of $\alpha6\beta4$ and BM-600/nicein in BCC cells but not in SCC cells indicates that $\alpha6\beta4$ integrin and one of its potential ligands may be co-regulated in both BCC and SCC, thus suggesting a role for this phenomenon in the pathogenesis and clinical behavior of these epidermal tumors. Key words: squamous cell carcinoma/basal cell carcinoma/integrins/basement membrane proteins. *J Invest Dermatol 101:352–358, 1993*

Basal cell carcinomas (BCCs) are cutaneous tumors, exclusively localized in hairy skin, especially of the face. BCCs do not metastasize and are only locally invasive, although metastases are exceptionally observed [1]. The typical cells of BCC resemble basal epidermal keratinocytes; this suggests that the latter are the cells where transformation occurs, giving rise to BCC. Tumor cells usually form well-demarcated aggregates. However, the surrounding stroma, arranged in parallel fibrous bundles around the tumor masses and crowded with numerous young fibroblasts [2], often shows infiltrative growth. Stroma retraction frequently occurs around tumor islets, resulting in peritumoral lacunae that may originate from the degeneration of palisade peripheral cells [3]. Immunostaining of the epidermal basement membrane zone (BMZ) has shown the presence of laminin and type IV collagen around the tumor as well as along the stromal side of the lacunae [4,5]. Data on the topography of bullous pemphigoid (BP) antigens are still unclear; some authors have found a variable and faint expression of BP antigen [6,7], whereas others have reported the absence of BP antigen in BCC [5,8]. To our knowledge, no information is available on other BMZ components.

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Reprint requests to: Dr. Pier Carlo Marchisio, Dipartimento di Scienze Biomediche e Oncologia Umana, Università di Torino, C.so Massimo d'Azeglio 52, 10126 Torino, Italy.

Abbreviations: BCC, basal cell carcinoma; BP, bullous pemphigoid.

Squamous cell carcinoma (SCC) may occur anywhere in the skin and in mucous membranes lined with squamous epithelia. It is a highly invasive and metastasizing carcinoma of the surface integument [9] consisting of irregular masses of anaplastic squamous cells that rapidly proliferate and deeply penetrate the underlying dermis. A characteristic feature, mostly observed in well-differentiated histotypes, is the presence of horn pearls, composed of concentric layers of squamous cells showing gradually increasing keratinization toward the center. Usually, the invading masses are poorly demarcated from the surrounding stroma, and the basal layer appears disorganized. Nevertheless, well-differentiated SCC express all BMZ components [8].

Several classes of molecules are involved in cell-to-cell and cell-to-matrix interaction. Among these, integrins, a superfamily of transmembrane glycoprotein α/β heterodimers (reviewed in [10,11]) play a major role in defining adhesion-dependent polarity of epithelial cells [12,13]. Keratinocytes both *in vivo* and *in vitro* express and expose the integrins $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta4$ [12,14–16]. The heterodimers $\alpha2\beta1$ and $\alpha3\beta1$ are mostly located at the lateral surface of basal and immediately suprabasal keratinocytes, suggesting their role in cell-to-cell interaction [12,13,17,18], whereas $\alpha6\beta4$, sharply restricted to the basal domain of basal keratinocytes [12], is a potential major linker of the epidermis with the BMZ, presumably for its association to hemidesmosomes [16,18–20].

In this paper we report that i) the expression of integrin $\alpha6\beta4$ is lost in BCC but is highly conserved and homogeneously distributed over the whole cell surface in SCC; ii) islets of BCC tumor cells are surrounded by a BMZ-containing laminin and type IV collagen but

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not BM-600/nicein; iii) SCC cell masses display a BMZ-containing laminin, collagen type IV, and BM-600/nicein; iv) in both types of tumors, $\alpha2\beta1$ and $\alpha3\beta1$ are no longer polarized to the lateral boundaries but are expressed pericellularly. The simultaneous loss of $\alpha6\beta4$ and BM-600/nicein in BCC, but not in SCC, suggests that secretion and exposure of these molecules are co-regulated and may be involved in the peculiar pathogenesis and clinical behavior of these epidermal tumors.

MATERIALS AND METHODS

Tissues We examined 15 surgical samples of BCC and five of SCC. One half of each excised tumor was fixed in formalin and processed for routine histology. The other half was directly embedded in OCT 4583 embedding compound (Miles Scientific, Naperville, IL) and snap frozen. Five-micrometer frozen serial sections were cut in a Reichert-Jung cryomicrotome, transferred onto microscope slides coated with poly-L-lysine (Sigma Chemical Company), air-dried, and stored overnight at room temperature.

Antibodies The primary monoclonal antibodies (MoAbs) used in this study, with the investigators that kindly provided them, were as follows: TS2/7, to $\alpha1$ [21], B5G10, to $\alpha4$ [22], and A1-A5, to $\beta1$ [23], from M. Hemler, Dana Faber Cancer Institute, Boston, MA; 12F1, to $\alpha2$ [24], from V. Woods, University of California, San Diego, CA; F1 and F2, to $\alpha3$ [Kaczamarek *et al*, submitted] from L. Zardi, IST, Genova, Italy; MAR6, to $\alpha6$, from S. Ménard, INT, Milano, Italy; GOH3 to $\alpha6$ [25], from A. Sonnenberg, Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands; AA3 to $\beta4$ [26] from V. Quaranta, Scripps Research Institute, La Jolla, CA; 3E1 to $\beta4$ [27] from E. Engvall, La Jolla Cancer Research Foundation, La Jolla, CA; GB3 to BM-600/nicein [28] from P. Verrando, Laboratoire de Recherches Dermatologiques, UER Médecine, Nice, France. MoAbs SAM-1, to $\alpha5$ and Gi9, to $\alpha2$, were from Immunotech, Marseille, France; 4B4, to $\beta1$, was from Coulter Immunology, Hialeah, FL. Rabbit polyclonal antisera 5710 to $\beta4$ and 6842 to $\alpha6$ were generously provided by V. Quaranta, and rabbit antisera to laminin and collagen type IV were, respectively, from Sigma Chemical Company, St. Louis, MO, and Heyl GmBH, Berlin, Germany. Finally, MoAb IST9, to cellular fibronectin [29] and BC2 and BC4, to tenascin, were kindly provided by L. Zardi, IST, Genova, Italy.

The MoAbs were used at a final Ig concentration of 40 μg/ml; the polyclonal antibodies were diluted to a final concentration of 10 μg/ml.

Indirect Immunoperoxidase Technique Cryostat sections were fixed for 10 min in a chloroform-acetone mixture (1:1), air dried, and incubated for 10 min in phosphate-buffered saline (PBS) supplemented with 1% serum of the same animal species that were the source of the primary antibody. Serial sections were overlaid with 50 μl of the primary antibody at a concentration of 10–40 μg/ml in Hanks' balanced salt solution (HBSS) and incubated at room temperature for 30 min in a water-saturated chamber. After a thorough wash in PBS, the sections were incubated with the appropriate biotinylated secondary antibodies and processed for the ABC method (avidin-biotin-peroxidase complex) using the Vectastain ABC Kit (Vector Laboratories Inc., Burlingame, CA). After several washes, 100 μl of substrate were added for 5–10 min. The substrate was prepared as follows: 5 mg 3 amino-9 ethylcarbazole (Sigma) was dissolved in 1 ml N,N-dimethylformamide (Merck, Darmstad, Germany) supplemented with 9 ml 100 mM sodium acetate, pH 5.2, and 100 μl 12% H_2O_2. All samples were counterstained with Mayer's hemalum solution, mounted in Kaiser's glycerol gelatin (Merck), and examined with a Zeiss Axiophot photomicroscope equipped with 16× and 63× planapochromatic lenses.

Cell Cultures FG pancreatic adenocarcinoma cells, a gift of V. Quaranta, Scripps Research Institute, La Jolla, CA, were cultured in RPMI 1640 medium (Biochrom KG, Berlin, Germany), supplemented with 10% fetal calf serum (Biochrom KG), 1% glutamin, and a 1% mixture of antibiotics, maintained at 37°C in a water-saturated atmosphere of 95% air–5% CO_2.

Immunoblotting Cultured pancreatic adenocarcinoma FG cells and frozen serial sections of BCC, SCC, and normal epidermis were solubilized in boiling Laemmli buffer [30] and sonicated. Equal amounts of proteins (300 μg) were loaded in all lanes. After sodium dodecylsulfate–polyacrylamide gel electrophoresis under non-reducing conditions, proteins were transferred to nitrocellulose filters (Hybond, Amersham Co, Arlington Heights, IL) and analyzed as described [31]. Filters were then probed with the polyclonal antibodies R5710 to $\beta4$ and R6842 to $\alpha6$. Specific binding was detected by the enhanced chemiluminescence system (Amersham).

RESULTS

Immunochemical Staining Immunoperoxidase staining was used to detect α and β chains of the integrin subfamily $\beta1$, the $\beta4$ chain, and the BMZ proteins laminin, type IV collagen, BM-600/nicein, and fibronectin in frozen serial sections of 15 BCC and five SCC surgical samples belonging to several histologic subtypes. No significant difference in immunochemical patterns could be related to a defined histotype.

The boundary around islets of BCC cells, as well as the BMZ of normal epidermis, was lined by type IV collagen and laminin (Fig 1*A,C*). Type IV collagen and laminin were also detected in the wall of peritumoral lacunae. Moreover, fibronectin and tenascin were abundant in the peritumoral stroma, according to their reported increased expression in cutaneous tumors.

The integrin chains $\alpha1$ and $\alpha4$ were absent from BCC cells, as well as from normal keratinocytes. The $\alpha5$ chain was virtually negative in BCC cells and in normal epidermis far from the tumor; instead, $\alpha5$ was markedly positive in the epidermis overlying the tumor and notably at sites where BCC cells occasionally showed infiltrative behavior (not shown).

Expression of heterodimers $\alpha2\beta1$ and $\alpha3\beta1$ was always detectable in BCC cells. The $\beta1$ chain was distributed over the entire surface of all BCC cells (Fig 2*A*) and its pattern was identical when both $\beta1$ MoAb were used (see *Materials and Methods*). It must be noted that the peripheral cell layer of the tumor masses, organized as a palisade, showed more intense staining at the basal surface.

The membrane expression of the $\alpha2$ subunit was uniform on the whole surface of all cells; in contrast the $\alpha3$ subunit was more markedly expressed by the peripheral cell layer. In BCC with areas of keratinization or keratotic differentiation, we observed significantly lower staining for $\alpha2\beta1$ and $\alpha3\beta1$ (Fig 2*C,E*).

A major finding of this investigation was the lack of expression of $\alpha6\beta4$ heterodimer on BCC cells. The $\beta4$ chain was virtually absent (Fig 3*A,C*) whereas $\alpha6$ was sometimes faintly expressed around aggregates of tumor cells (Fig 3 *E,G*). The distribution of BM-600/nicein exactly matched the pattern displayed by $\alpha6\beta4$. In fact, although BM-600/nicein was detected in a linear pattern at the BMZ of the epidermis overlying the tumor, it was completely undetectable around aggregates of neoplastic cells (Fig 1*E*). Where aggregates of tumor cells were in continuity with normal epidermis, there was a sharp transition between normal BMZ, displaying BM-600/nicein, and the tumoral BMZ, where both $\beta4$ and BM-600/nicein were immunohistochemically undetectable. However, each aggregate of BCC cells was surrounded by a BMZ-containing laminin and type IV collagen (Fig 1*A,C*).

As expected, $\beta4$ sharply lined the basal surface of basal keratinocytes in the normal epidermis overlying the tumor. In contrast, at sites where aggregates of tumor cells were in contiguity or infiltrated the epidermis, $\beta4$ lost its basal distribution and was also detected on the lateral surface of epidermal keratinocytes (Fig 4*A,B*). Two different MoAbs to $\beta4$, presumably recognizing different epitopes, gave identical patterns.

In contrast, in all samples of SCC, $\alpha6\beta4$, as well as $\alpha2\beta1$ and $\alpha3\beta1$, were strongly expressed by the peripheral cells of invading tumor masses (Fig 3*B,D,F,* and *H*). In SCC we observed a complete loss of polarized integrin topography. In fact, pericellular staining for $\alpha2\beta1$, $\alpha3\beta1$ (Fig 2*B,D,* and *F*), and $\alpha6\beta4$ was observed on the membrane of all peripheral SCC cells. Furthermore, SCC cells, but not BCC cells, produced and exposed the fibronectin receptor $\alpha5\beta1$ whereas $\alpha1$ and $\alpha4$ integrin chains were negative on SCC cells, as well as on BCC cells and normal keratinocytes.

Although invading SCC cell masses seemed to be poorly demarcated from the surrounding stroma, no specific defect in BMZ components could be detected; this finding was in marked contrast with the above-reported defects of BMZ in BCC. In fact a marked depo-

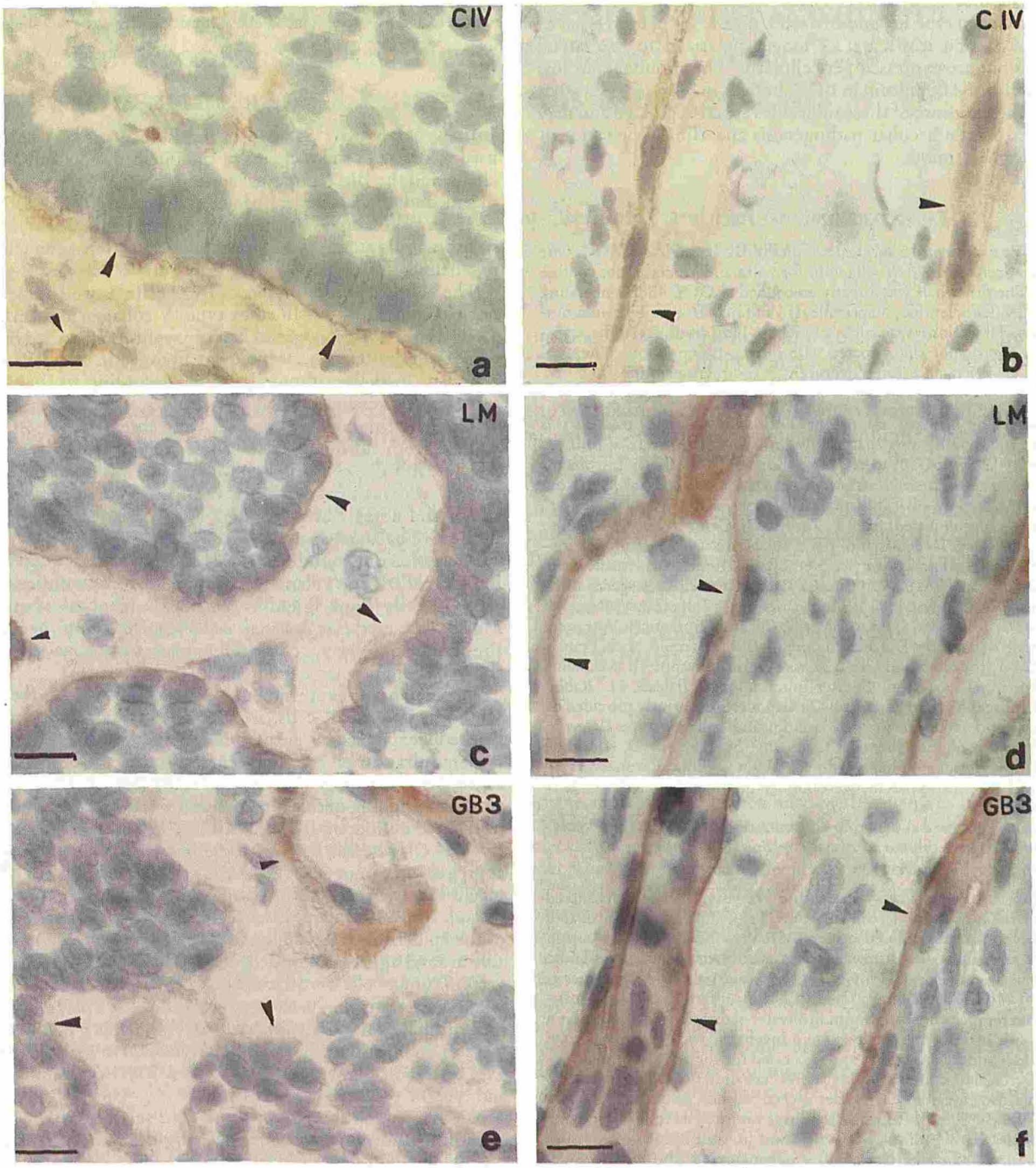

Figure 1. Immunoperoxidase staining of BCC *(left column)* and SCC *(right column)*; the antibody binding was visualized using the avidin-biotin–conjugated peroxidase Vectastain ABC kit. In BCC, type IV collagen *(a)* and laminin *(c)* were highly expressed around aggregates of tumor cells, whereas no staining of BM-600/nicein *(e)* was observed. In SCC all three BMZ proteins *(b, d, and f)* were similarly detectable. *Bar, 3 μm.*

sition of type IV collagen (Fig 1*B*) and laminin (Fig 1*D*) was found around the tumor, as was high positivity for BM-600/nicein (Fig 1*F*), showing the same linear pattern detected at epidermal BMZ. Fibronectin and tenascin were also very abundantly secreted and organized in the SCC peritumoral stroma (not shown).

Western Blot Analysis To investigate whether the expression of α6β4 integrin in BCC and SCC was different from that of normal epidermis, Western blot analysis of extracts prepared from tumor tissues and normal epidermis was performed using the polyclonal antibodies R5710 to β4 and R6842 to α6. Positive controls were provided by extracts of pancreatic adenocarcinoma FG cells [26].

A 200-kD protein band corresponding to β4 chain was visualized by antibody R5710 in the lanes of FG cells (Fig 5, *lane D*), normal skin (Fig 5, *lane A*), and SCC (Fig 5, *lane B*), whereas no antibody R5710 reactivity could be found in the lane corresponding to BCC (Fig 5, *lane C*). Similarly, R6842 antibody decorated a 150-kD band corresponding to the expected α6 band in samples positive for β4 but resulting negative on BCC (Fig 5, *lanes E–H*). The differential

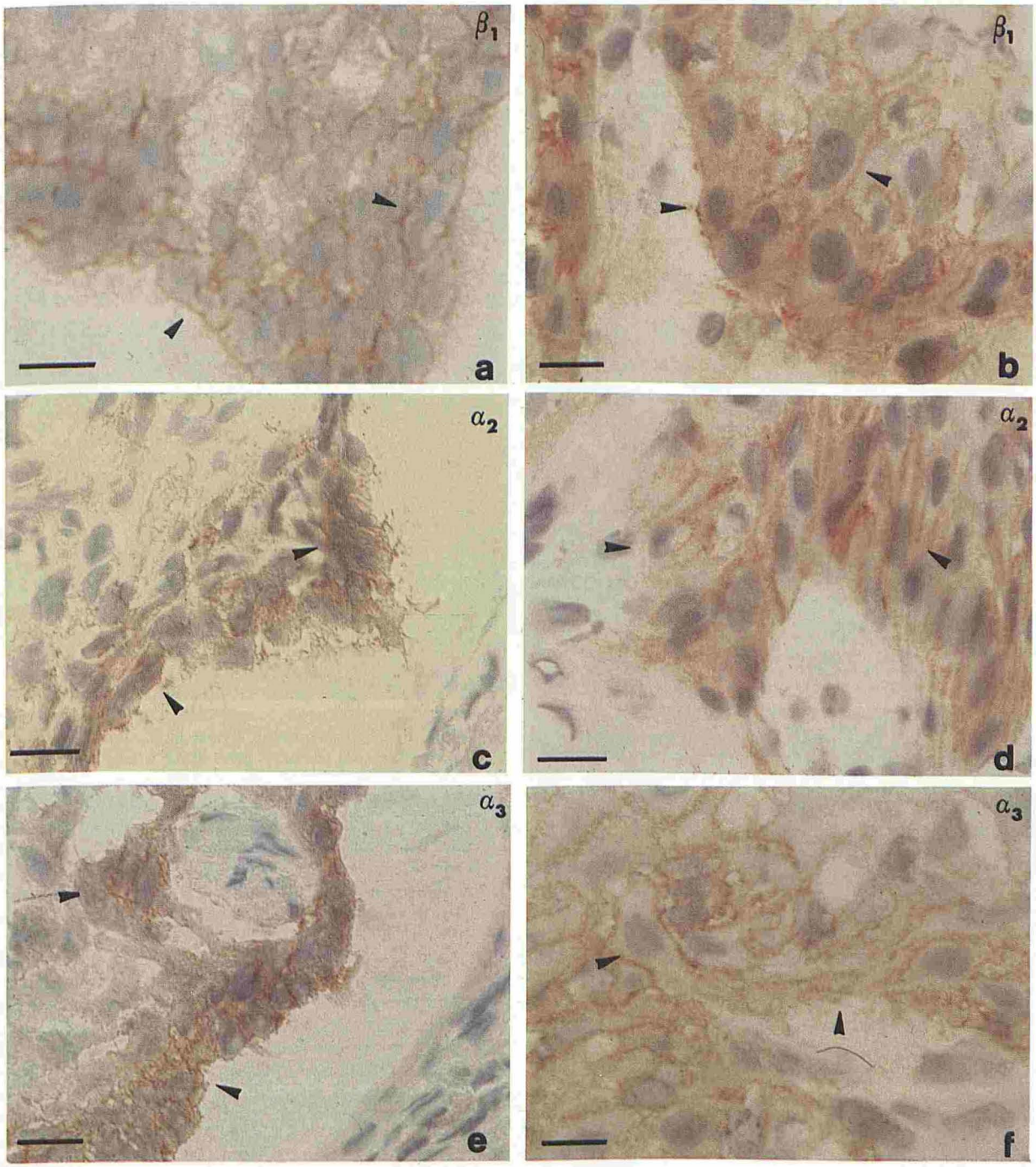

Figure 2. Immunoperoxidase staining of BCC *(left column)* and SCC *(right column);* in both tumors the integrins $\alpha 2\beta 1$ *(a–d)* and $\alpha 3\beta 1$ *(a,b,e,f)* were conserved but showed a perturbed topography and a loss of polarization, being aspecifically distributed all along the cell margins. *Bar,* 3 μm.

expression of $\alpha 6\beta 4$ integrin heterodimer in different cutaneous carcinomas was then confirmed by Western blot analysis.

DISCUSSION

In this study we show that BCC and SCC, two cutaneous tumors characterized by different degrees of malignancy, display abnormal yet different patterns of integrin expression. First, a change occurs in the localization of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ heterodimers. In normal keratinocytes, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins are located to the lateral surface of basal and immediately suprabasal cells [12,17,18], but in both BCC and SCC the polarized distribution of these integrins is modified and replaced by pericellular diffusion. We cannot confirm whether there is higher expression of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in BCC than in basal epidermal cells [32], as our immunohistochemical data are not quantitative and simply show altered topography of integrins. However, altered topography of integrins is not peculiar to epidermal neoplasias. In fact, such alteration has also been observed, without any obvious increase of integrin synthesis, in psoriasis, a cutaneous disease characterized by abnormal cell proliferation and maturation [33].

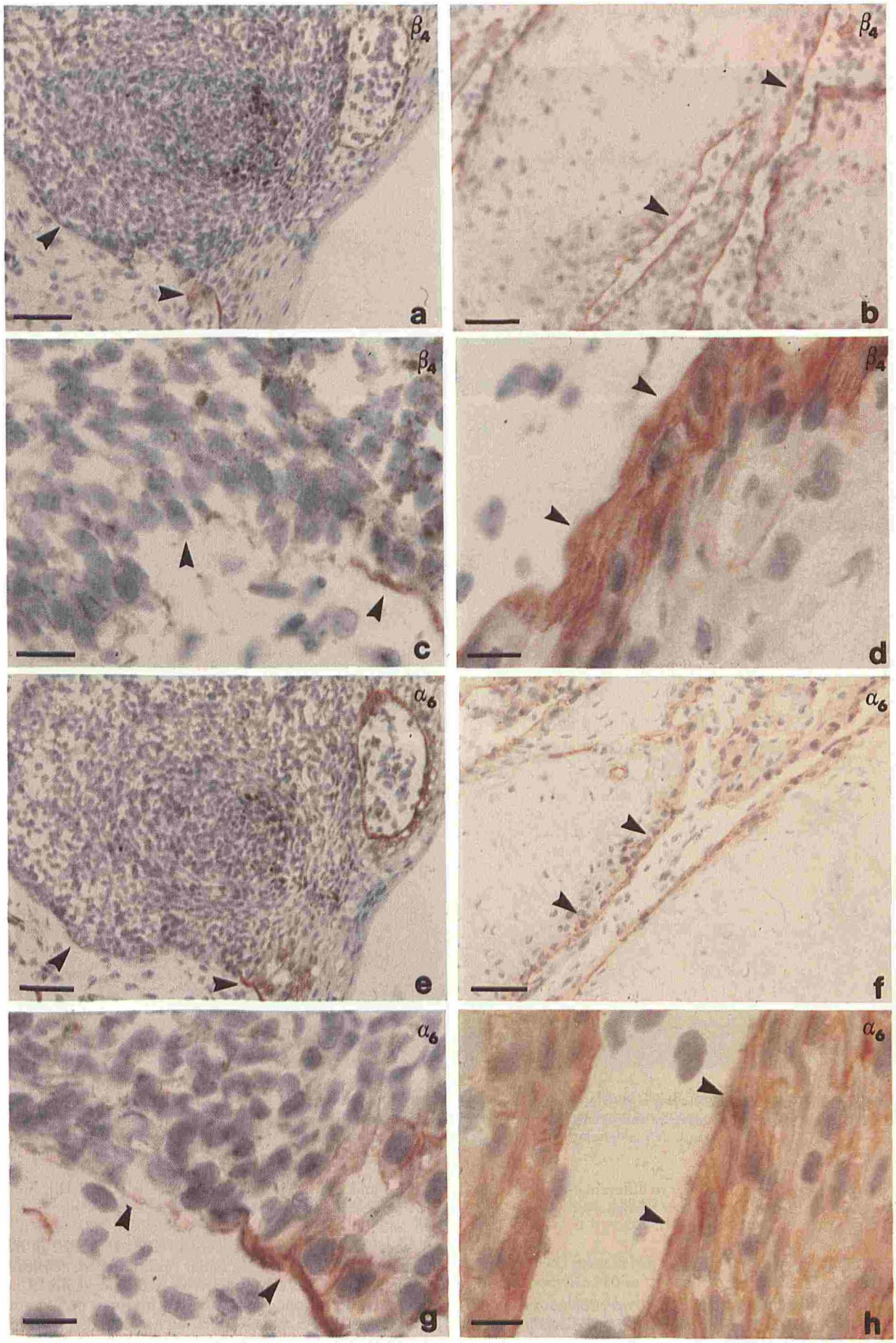

Figure 3. Immunoperoxidase staining of BCC *(left column)* and SCC *(right column)*; in BCC the $\beta 4$ chain was absent *(a,c)*, whereas $\alpha 6$ was only faintly expressed around tumor cell aggregates *(e,g)*. In SCC $\beta 4$ *(b,d)* and $\alpha 6$ chains *(f,h)* were strongly expressed by the peripheral cells of invading tumors masses and, similarly to $\alpha 2\beta 1$ and $\alpha 3\beta 1$, showed a complete loss of polarized topography. *Bar,* 10 μm *(a,b,e,f)*. *Bar,* 3 μm *(c,d,g,h)*.

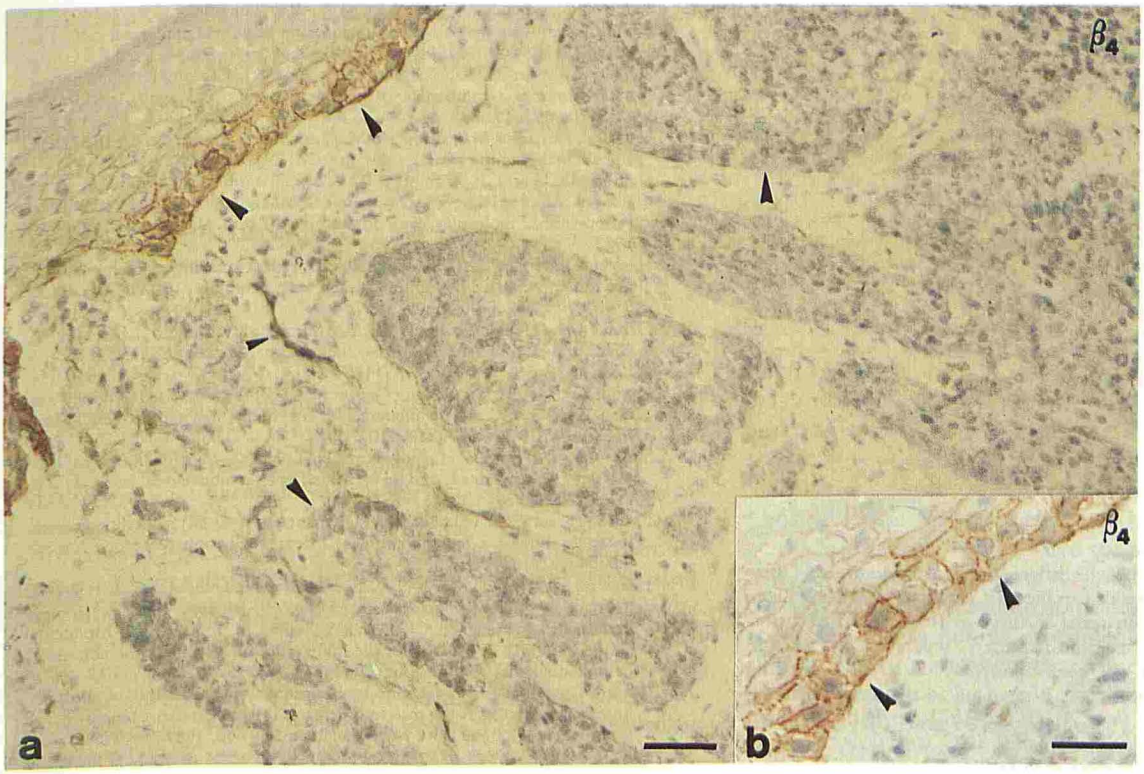

Figure 4. Immunoperoxidase staining of BCC; in normal epidermis overlying tumor masses, the $\beta 4$ chain lost its polarized distribution and was also present on the lateral surface of keratinocytes *(a,b)*. *Bar,* 10 μm *(a); Bar,* 3 μm *(b)*.

The second major change is the loss of $\alpha 6$ and $\beta 4$ integrin expression in BCC cells shown by Western blotting and by immunohistochemistry. In rare cases a faint persistence of $\alpha 6$ expression was noted and could be related to the possible expression of minute amounts of the $\alpha 6\beta 1$ heterodimer that is not normally assembled when the $\beta 4$ chain is synthesized [12]. In normal keratinocytes, $\alpha 6\beta 4$ is localized at hemidesmosomes [16,18,19] and contributes to matrix adhesion [12,35], although the ligand of the $\alpha 6\beta 4$ complex has not yet been identified in any defined laminin domain [36]. However, it is generally agreed that the $\alpha 6\beta 4$ ligand will be identified in a molecular component of the matrix deposited by cultured human keratinocytes where a coherent distribution of $\alpha 6\beta 4$ and laminin has already been shown [13]. Other candidate ligands of the $\alpha 6\beta 4$ complex are the recently identified BMZ proteins epiligrin [37], kalinin [38], and BM-600/nicein [28,39]. Epiligrin co-distributes with $\alpha 6\beta 4$-containing stable anchoring complexes in cultured keratinocytes, but it has been suggested to be a ligand for $\alpha 3\beta 1$ on the basis of the activity of function-blocking antibodies [37]. Kalinin has been located mostly in hemidesmosomes and may be a molecular link between anchoring fibrils and the $\alpha 6\beta 4$-containing hemidesmosomal plate [38]. In normal skin, BM-600/nicein lines the basal surface of basal epidermal cells [40,41] and corresponds to the position of $\alpha 6\beta 4$. BM-600/nicein is severely altered in patients suffering from a lethal form of epidermolysis bullosa [41], and this finding suggests that this protein may play a relevant role in linking the epidermis to the underlying dermis. Recent data suggest that the above molecules are indeed identical [42].

A third point is that the lack of $\alpha 6\beta 4$ in BCC cells corresponds to the persistence of a continuous BMZ containing laminin and type IV collagen, which surrounds tumor cell islets. However, the BMZ of BCC is defective because BM-600/nicein is missing around tumor islets. Conversely, in SCC, although the invading cell masses are poorly demarcated from the surrounding stroma, the expression of BM-600/nicein, laminin, and type IV collagen is similar to that of normal epidermis.

At variance with BCC, $\alpha 6\beta 4$ is expressed at high levels by peripheral cells of SCC aggregates but its topography is severely altered and similar to the pericellular distribution of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins. This finding confirms that loss of integrin polarity indeed occurs in SCC. Increased levels of A9 expression, a glycoprotein complex with structural and functional features of the $\alpha 6\beta 4$ integrin [43], was indeed reported in SCC lines [44] long before $\alpha 6\beta 4$ was discovered.

The major result of this investigation is the complete absence from BCC cells of $\alpha 6\beta 4$ and of one of its potential ligands. To our knowledge, this is the first report of an epithelial neoplasm characterized by the simultaneous absence of an integrin and its potential ligand. This finding has a few implications. First, $\alpha 6\beta 4$ and BM-600/nicein may have comparable behavior and may be regulated by a common mechanism, thus supporting the concept that the two molecules face each other at the basal aspect of basal epidermal keratinocytes [28]. This concept is corroborated by the fact that $\alpha 6\beta 4$ and BM-600/nicein are codistributed in primary normal keratinocytes in culture [45], where they form a molecular assembly, indicating a ligand-receptor relationship. A second consideration is that the BMZ of epithelial neoplasms may be defective in one single component whereas the others are preserved. Whether this event is related to malignancy remains to be proved. SCC, where most integrins and BMZ molecules are expressed, are associated with metastatic spreading. On the contrary, the simultaneous absence of $\alpha 6\beta 4$ and BM-600/nicein in the BCC adhesive phenotype could be related to the prevention of metastatic spreading.

In conclusion we propose that i) simple expansive growth is associated with the specific absence of the basal integrin and one BMZ molecule; ii) the capacity of metastatic diffusion in epidermal cancers correlates with the preservation of basal membrane receptors and their potential matrix ligands; and iii) loss of controlled cell proliferation is associated with loss of integrin polar topography in neoplastic as well as in psoriatic keratinocytes [33].

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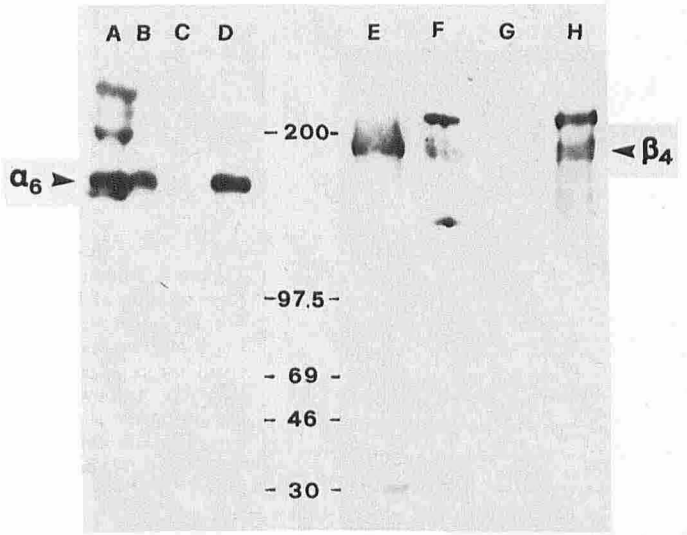

Figure 5. Western blot analysis of the differential expression of $\alpha 6\beta 4$ integrin complex in different cutaneous carcinomas; proteins were solubilized from cultured FG cells or frozen serial sections of BCC, SCC, and normal epidermis with boiling Laemmli buffer, separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis, under non-reducing conditions, transferred to nitrocellulose filters, and incubated with rabbit polyclonal antisera R6842 to $\alpha 6$ *(lanes A–D)* and R5710 to $\beta 4$ *(lanes E–H). Lanes A and H,* normal epidermis; *lanes B and F,* SCC; *lanes C and G,* BCC; *lanes D and E,* pancreatic adenocarcinoma FG cells.

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